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ORIGINAL PAPER

Medium, container and genotype all influence in vitro cold storage of apple germplasm

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Abstract The goal of this study was to evaluate the in vitro storage of apple germplasm by screening a range of genotypes followed by more comprehensive testing of multiple parameters on two genotypes of differing species, *Malus domestica* cultivar Grushovka Vernenskaya and wild *Malus sieversii* selection TM-6. Stored plants were rated on a 6 point scale (0 low to 5 high) for plant appearance at 3 month intervals after storage at 4°C. Combinations of carbon source (sucrose and/or mannitol), nitrate nitrogen content (25, 50 or 100%) and plant growth regulators (ABA, BAP, IBA) were studied in three types of containers (tissue culture bags, test tubes or jars). An initial screen of 16 genotypes stored in tissue culture bags indicated that plantlets could be stored at 4°C for 9–14 months without subculture on standard 3% sucrose Murashige and Skoog (1962) (MS) medium with no plant growth regulators (PGRs). In subsequent in-depth studies on the two genotypes, ANOVA indicated highly significant interactions of medium, container and genotype. 'Grushovka Vernenskaya' shoots with no PGRs and 3% sucrose remained viable (ratings of $\geq$1) for 21 months of storage in bags. Storage on reduced nitrogen (MS with 25% nitrogen), PGRs, and 3% sucrose kept 'Grushovka Vernenskaya' shoot condition rated >2 at 21 months. Addition of 0.5 or 1 mg^{-1} abscisic acid (ABA) also improved plant ratings at 21 months. The longest storage for 'Grushovka Vernenskaya' was 33–39 months with PGRs and 3% sucrose in either tubes or jars. Addition of abscisic acid (ABA) to the medium did not improve storage of plantlets in jars and tubes at 15 months. TM-6 stored best in tubes on 3% sucrose with PGRs or in jars on 2% mannitol and 2% sucrose. Overall it appears that cold storage of apple shoot cultures can be successful for 21 months in tissue culture bags with 25% MS nitrate nitrogen, 3% sucrose, and no PGRs or for 33 months in jars or tubes on MS with 3% sucrose and PGRs. Preliminary RAPD analysis found no significant differences between plants stored for 39 months and non-stored controls.

Keywords In vitro storage · *Malus domestica* · *Malus sieversii* · Micropropagation · Germplasm storage

Abbreviations

ABA	Abscisic acid
BAP	Benzylaminopurine
IBA	Indole 3 butyric acid
PGR	Plant growth regulator

Introduction

Apple (*Malus domestica* L.) is the most widely cultured fruit crop in temperate zones. Development of apple cultivars resistant to abiotic and biotic stresses (diseases and insects) is necessary for maintaining economically viable orchards. The raw material for development of new cultivars is dependent on genetic resource collections such as those

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found in the Pomological Garden at the Institute of Horticulture and Viticulture near Almaty, Kazakhstan, a center of genetic diversity for apples. The apple collection of the pomological garden contains 1667 cultivars and 3 species with 60 representative cultivated genotypes and wild selections. About 50 genotypes are held in vitro. Cold storage of the in vitro collection provides additional security while keeping the plants available for study or distribution. In vitro culture and cold storage are secondary-storage methods for large plant germplasm collections (Ashmore 1997).

Plantlets held in cold storage at around 4°C can remain without reculture for several years. This type of storage is effective for many temperate crops, including small fruit and tree fruit (Ashmore 1997; Reed and Chang 1997; Reed et al. 1998; Wanas et al. 1986). There are several reports of apple shoot cultures stored in vitro. These reports are limited to one or two genotypes per study and there are no reports of the application of this technique to large germplasm collections. The first report of successful in vitro cold storage was with the apple 'Golden Delicious', in test tubes at 1 or 4°C in the dark; shoots remained in good condition for 1 year, with some losses to contamination (Lundergan and Janick 1979). In another study, two apple rootstocks 'P2' and 'M. 9' that were stored for 48 weeks in the dark at 4°C remained in the best condition when grown in tubes on medium with growth regulators and when the explants stored were multi-branched tufts rather than single shoots (Orlikowska 1992b). Negri et al. (2000) studied single node cuttings of two genotypes stored at 4°C in the dark for 18 months in small tubes. They found genotype differences and growth regulator effects. A moderate amount of N^6 benzyladenine (BA) supported the best storage, although hyperhydricity was a problem for some plants (Negri et al. 2000). Single bud lines of 'Gala' apple, stored for 1 year at 4°C with a 14-h photoperiod, were studied for genetic variation. The ploidy remained stable and AFLP analysis with 20 primer pairs showed no differences in the plants before and after storage, however, some methylation changes were noted in the stored shoots (Hao and Deng 2003). Apple rootstock 'M. 26' stored well for 9 months in gas-permeable bags or 500 ml glass jars at 4°C in the dark (Lambardi et al. 2006). These studies show the potential of in vitro cold storage for use in preserving apple germplasm, but they also indicate that there may be variability due to the genetic diversity of large collections.

This study evaluated the in vitro storage of a range of diverse apple genotypes as well as an in depth study of two selected genotypes: a cultivar and a wild Kazakhstan selection. The effects of genotype, growth regulators, nitrogen, carbohydrates, and container type on cold-storage duration were studied. RAPD analysis of the plants was performed before and after storage to detect possible genetic variation. The overall goals of this study were to define parameters for the medium-term storage of diverse genotypes, and to apply the results to the wider germplasm collection.

Materials and methods

Plant material

Apple [*Malus domestica* L. and *Malus sieversii* (Ledeb.) M. Roem] genotypes were micropropagated in 130 ml glass jars with 10 ml MS medium with 1.0 mg mgl^{-1} N^6 benzylaminopurine (BAP), 0.5 mg mgl^{-1} indole-3-butyric acid (IBA) and 0.7% agar at pH 5.7. Plantlets (3 cm) were cultured in 10 ml of MS medium in 130 ml glass jars at 24°C with a 16-h light photoperiod (25 μmol m^{-2} s^{-1}) and subcultured on a 3–4 week cycle prior to storage.

Storage conditions

Shoots (3 cm) from micropropagated plants were subcultured onto 10 ml MS medium with 0.8% agar without growth regulators in 50 ml glass tubes, 130 ml glass jars and semi-permeable plastic tissue-culture bags (Star Pac Bags, PhytoTechnology Laboratories, Shawnee Mission, Kansas). Plantlets in the storage containers were placed in the growth room for 1 week and then cold acclimated for 1 week at 8-h light 23°C/16-h dark, 4°C before being placed in storage at 4°C with a 10-h light photoperiod with low irradiance (7–15 μmol m^{-2} s^{-1}) (Reed 1991).

Cold-storage screen of germplasm in tissue culture bags

General observations of growth condition were taken monthly for 15 months on 16 apple genotypes stored in tissue culture bags on 10 ml MS medium with no growth regulators. Replication was 5 plantlets per genotype (5 sections of one storage bag) for this screen and it was not repeated. Apple genotypes (cultivars and selections from wild plants) used in this screen were: 'Agat', 'Aport', 'Damira', 'Golden Delicious', 'Grushovka Verneskaya', 'Makpal', N 36, N 43, 'Naggit', 'Nurgul', 'Starkrimson', 'Royal Red Delicious', 'Syislepper', TM-6, 'Ulan', and 'Voskhod'. The percentage of living plants for each genotype was noted at each rating period.

Cold-storage studies of *Malus domestica* 'Grushovka Vernenskaya' and *Malus sieversii* selection TM-6

The effect of growth regulators, carbohydrates, vessel volume and genotypes

Duration of in vitro cold storage and plant condition under a variety of storage parameters were examined for

'Grushovka Vernenskaya' and TM-6. These were chosen to represent the two species and two levels of native cold hardiness; moderate for 'Grushovka Vernenskaya' and deep for TM-6. All treatments were done in triplicate ($n = 15$). Plantlets were stored in 50 ml tubes, 130 ml glass jars and air permeable plastic bags with 10 ml of MS medium dispensed in all containers. All plants were acclimated for 1 week and placed in storage as noted above. The four carbohydrate treatments tested were: 3% sucrose, 2% or 3% mannitol, and 2% sucrose plus 2% mannitol. Each treatment was with or without plant growth regulators (PGRs) 0.5 mg mgl^{-1} BAP and 0.1 mg mgl^{-1} IBA.

MS nitrate nitrogen at 100, 50 and 25% was tested in bags with or without PGRs and with 3% sucrose with 'Grushovka Vernenskaya'.

Abscisic acid (ABA) 0.1; 0.5 or 1.0 mg mgl^{-1} was tested on MS without PGRs, with 3% sucrose and in bags, tubes and jars for 'Grushovka Vernenskaya' and tubes and jars for TM-6.

Data collection

Stored plants were evaluated at 3 month intervals to study the condition and actual mortality of plantlets. Shoots were rated on the scale from 0 to 5, based on plant appearance (Reed 1992): 0—dead plant;1—plants are brown, in some places yellow; 2—plants are yellow-brown; 3—plants with etiolation, yellow green; 4—plants with little etiolation, green; 5—plants with bright green leaves and stems. Under normal medium-term storage the plants would be removed for repropagation when the rating reaches 2.

Data analysis

The initial screen of germplasm was not replicated and had 5 plants for each genotype. In each medium-alteration experiment 5 plantlets in jars or 5-section bags and 5 plantlets in individual tubes were used for each genotype with 3 replicates ($n = 15$). Data was analyzed by ANOVA and means analysis with SYSTAT version 12 (SYSTAT 2007).

Molecular analysis

Genetic variability of cold-stored apple samples was studied using RAPDs. Three samples were analyzed for 'Grushovka Vernenskaya' and TM-6 (1) prior to storage, and (2) after 39 months of cold storage in jars on MS medium with PGRs.

The leaves were homogenized in liquid nitrogen and DNA was extracted. The homogenate was lysed in buffer: 0.1 M EDTA, 0.1 M Tris–HCl, pH 8.5, 0.1 M NaCl, 1% SDS, 0.1% Triton X-100 and de-proteined in phenol-chloroform-isopropanol mixture. Purity of extracted DNA was checked in 0.6% agar gel.

Primers were synthesized on an ABI 3900 DNA synthesizer (Applied Biosystems, USA). The reaction mixture for RAPD-PCR of 20 µl contained: 50 mM KCl, 20 mM Tris–HCl, pH 8.4, 2–5 mM MgCl2, 0.01% Tween-20, on 0.2 mM of each dATP, dTTP, dCTP, dGTP, on 0.2 nM primer, 20 ng DNA and 1 unit of Taq-polymerase. In test tubes 20 µl of mineral oil was overlaid on the reaction mixture. Amplification was carried out using free primers of more than 15 nucleotides (6 primers in total). Reaction with free primers of 10 nucleotides was carried out: the first denaturation 95°C, 3.5 min; one cycle 39°C, 2 min; 72°C, 2 min; 94°C, 1 min; then 33 cycles 47°C, 1.5 min; 72°C, 2 min; 94°C, 1 min; the last elongation 72°C, 6 minutes. Amplification fragments were fractionated in 10% polyacrylamide gel in TBE-buffer at voltage 120 B for 6 h. The spectra were photo documented in UV light on a gel documenting system (Bio-Rad). Initially 23 primers were tested on 14 genotypes, six were further tested and finally four primers were selected for use with the cold-stored material. These primers were GC > 50% and they were randomly selected. Primers that gave amplification products of 30–40 bands were used.

Results

Apple genotypes stored in tissue culture bags (screening trial)

Storage of 16 wild and cultivated apples in bags on standard MS medium with no PGRs generally showed one of three responses (Fig. 1). In the first group with short storage (6 genotypes) the plants declined over 9–12 months and were below a rating of 1 at 8–12 months (Fig. 1a). In the second group with moderate storage (4 genotypes) the plant condition declined to a rating of about 2 at 6–9 months, and then produced new growth that remained in good condition for an additional 3–6 months (Fig. 1b). The third group with long storage (6 genotypes) had high ratings (2.5–4) for 9 months and then the plants gradually declined to a 2 rating at 12–15 months (Fig. 1c). The widest variation among genotypes was represented by 'Ulan' and 'Damira' that were rated 2 at 3 months (Fig. 1a), and by 'Makpal' and 'Naggit' that only declined to a 2 rating at 15 months (Fig. 1c). In practice, plants are removed from storage for repropagation when the mean rating is ≤ 2. A comparison of the plant rating with the percentage of live plants at 3, 6, 9 and 12 month rating periods validates this practice as "2" rated plants generally had 60% or greater viability and none had lost all viability (Fig. 2).

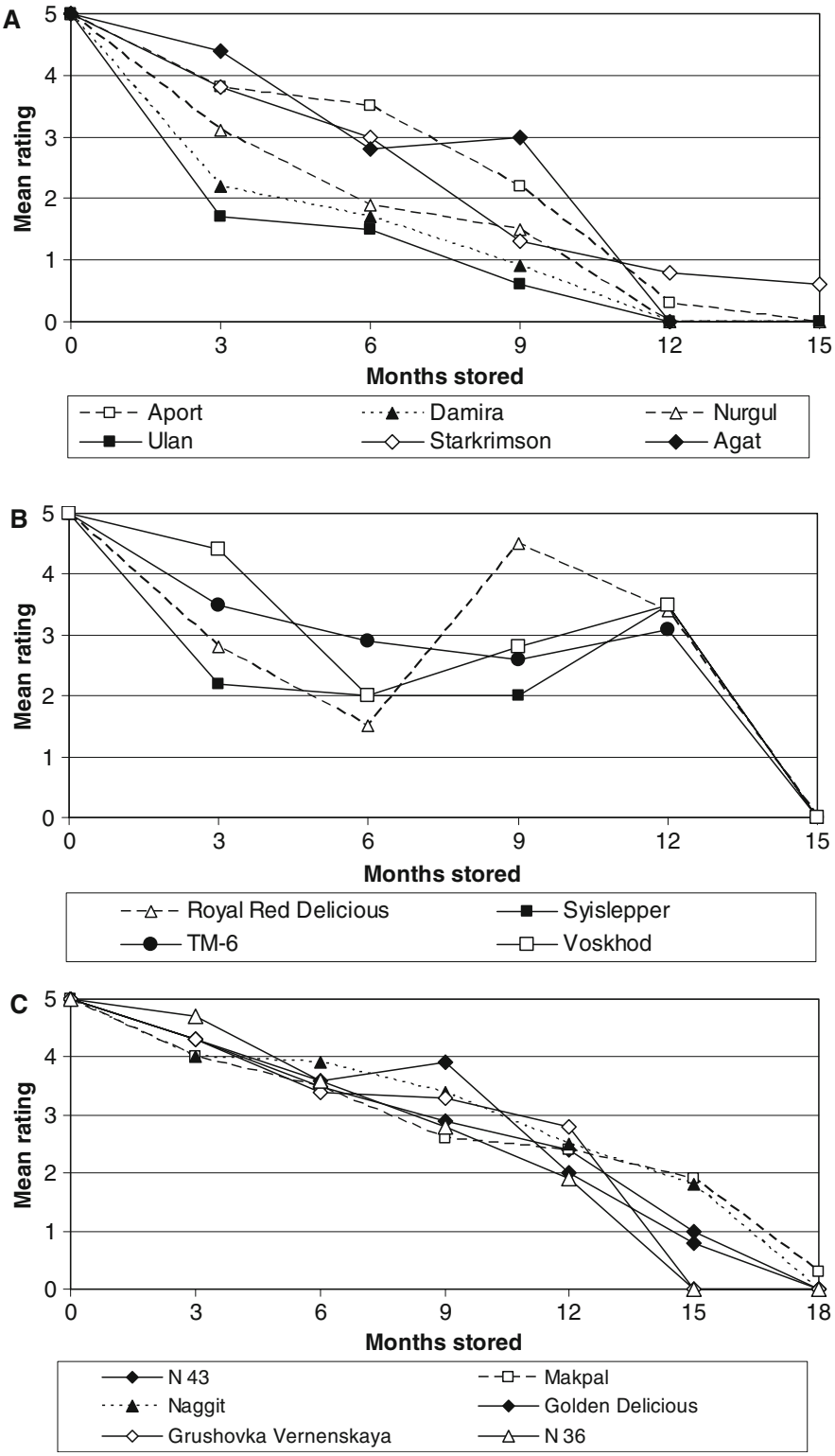

Fig. 1 Mean ratings of in vitro cultures stored at 4°C in tissue culture bags on MS medium with 3% sucrose and no PGRs (unreplicated screen). Each plant in a section of a 5 section bag was rated at 3 month intervals. Screened genotypes showed 3 storage responses: **a** rapid decline. **b** early rapid decline and regrowth with a second decline after 12 months. **c** gradual decline. Normally plants are removed from storage with a 2 rating

The effect of medium additives and container on storage duration

Two genotypes, 'Grushovka Vernenskaya' from the long storage category and TM-6 from the medium-storage category, were further tested over storage treatment combinations of 3 containers and 8 medium treatments (4 carbon sources with or without growth regulators). ANOVA of the 15 month data showed a significant interaction ($P < 0.001$) between container and medium

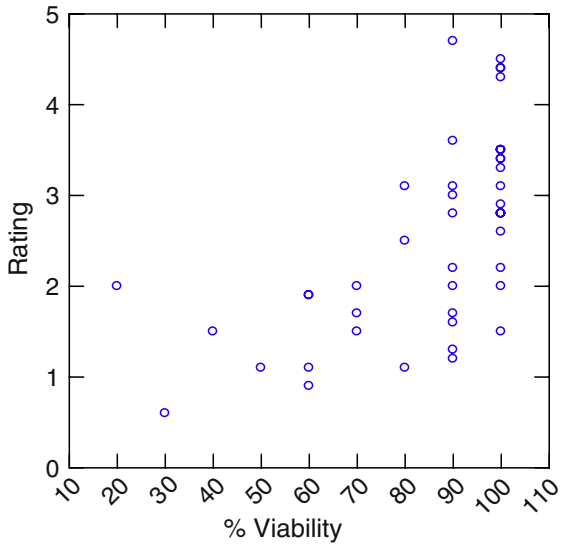

Fig. 2 Scatter plot comparison of mean condition ratings and % plant viability of the 16 apple genotypes shown in Fig. 1. Each point is one genotype at the 3, 6, 9 and 12 month rating periods. Only ratings >0 are shown and each viability % is based on 5 plants per genotype

treatments. The main effects of genotype, medium treatment and container were also significant. The poorest responses were on treatments with mannitol. Cultures stored on treatments with 2% or 3% mannitol declined quickly and reached a rating of 2 within 6–12 months with all three types of containers (Fig. 3). All plants stored on mannitol treatments were rated significantly lower ($P < 0.001$) than the controls on 3% sucrose with or without PGRs (Fig. 4). In bags the longest storage of 'Grushovka Vernenskaya' was only 6–9 months on 3% mannitol with PGRs (Fig. 3a). In tubes, all of the treatments produced similar results with about 6 months storage (Fig. 3b). The longest storage for 'Grushovka Vernenskaya' in jars was for 9 months on 3% mannitol with no PGRs while plants in the other treatments declined after 6 months (Fig. 3c). TM-6 cultures also declined rapidly on mannitol medium but remained in better condition for 2-3 months longer. Cultures in tubes and jars dropped below 2 ratings by 12 months (Fig. 3d, e).

'Grushovka Vernenskaya' shoots stored on medium with 3% sucrose or 2% sucrose and 2% mannitol remained viable (rating >1) in storage for 9–39 months depending on the treatment used (Fig. 4). Shoots stored in bags with 3% sucrose without PGRs remained in good condition (rating >2) for 9 months (Fig. 4a). At 15 months 3% sucrose or 2% sucrose and 2% mannitol treatments were significantly better than the other treatments ($P < 0.001$). On PGRs, these shoots declined faster in bags. Plants in both tubes and jars stored significantly better ($P < 0.001$) on 3% sucrose with PGRs (Fig. 4b, c) at 15 months than the other treatments. Storage in tubes with the other treatments declined within 9 months. Plants in jars on the other 3 treatments declined to a 2 rating at 9 months, but then grew new shoots from axillary buds and remained at ratings >2 for about 33 months. Plants on 3% sucrose with PGRs could be stored for about 3 years in jars or tubes.

TM-6 shoots stored in tubes on 3% sucrose with PGRs declined rapidly after 9–12 months, but then grew new shoots that remained in good condition (~3 rating) for 33 months (Fig. 4d). The 3% sucrose treatment with PGRs was significantly better than other treatments at 21, 30 and 33 months ($P < 0.01$). In tubes, shoots on 3% sucrose with no PGR treatment declined rapidly at 9–12 months and plants on sucrose plus mannitol treatments remained viable in storage (rating >2) for 18–24 months. In jars, the 2% mannitol plus 2% sucrose with or without PGRs had ratings >2 at 21 months (Fig. 4e). Cultures on 3% sucrose declined below the 2 rating at 6–9 months.

Effect of NO_3 on storage duration in tissue culture bags

Storage duration of 'Grushovka Vernenskaya' in bags with PGRs and 3% sucrose was greatly improved when NO_3 nitrogen was reduced to 25% of the normal MS level. After 12 months of storage the treatment with 25% NO_3 nitrogen and PGRs was rated significantly better ($P < 0.001$) than all other treatments (Fig. 5). 'Grushovka Vernenskaya' shoots were rated >2 at 21 months of storage on 25% N medium with PGRs and 3% sucrose. Shoots on all other treatments declined below a 2 rating at 12 months. Only the 25% NO_3 treatments with or without PGRs remained alive at 21 months and the PGR treatment was rated significantly higher than the no PGR treatment.

ABA effects on storage duration

Abscisic acid treatment interacted significantly with the culture container used and the genotype ($P < 0.001$). For 'Grushovka Vernenskaya' at 21 months, ABA at all concentrations significantly improved the condition rating of shoots stored in bags but decreased viability in jars and tubes (Fig. 6a). Cultures in jars without ABA were rated >2.5 at 21 months while those stored in tubes and bags were rated <1. Shoots in bags with either 0.5 or 1 mg mgl^{-1} ABA were rated 1 as compared to <1 without ABA. In contrast to 'Grushovka Vernenskaya', TM-6 stored best with 0.5 or 1 mg mgl^{-1} ABA in tubes, and 0.5 ABA was significantly better for shoots in jars (Fig. 6b).

Genetic analysis of cold stored cultures

Initial screening was used to select suitable primers. DNA analyses were initially carried out using RAPDs, RFLP analyses and DNA-DNA hybridization but only RAPDs

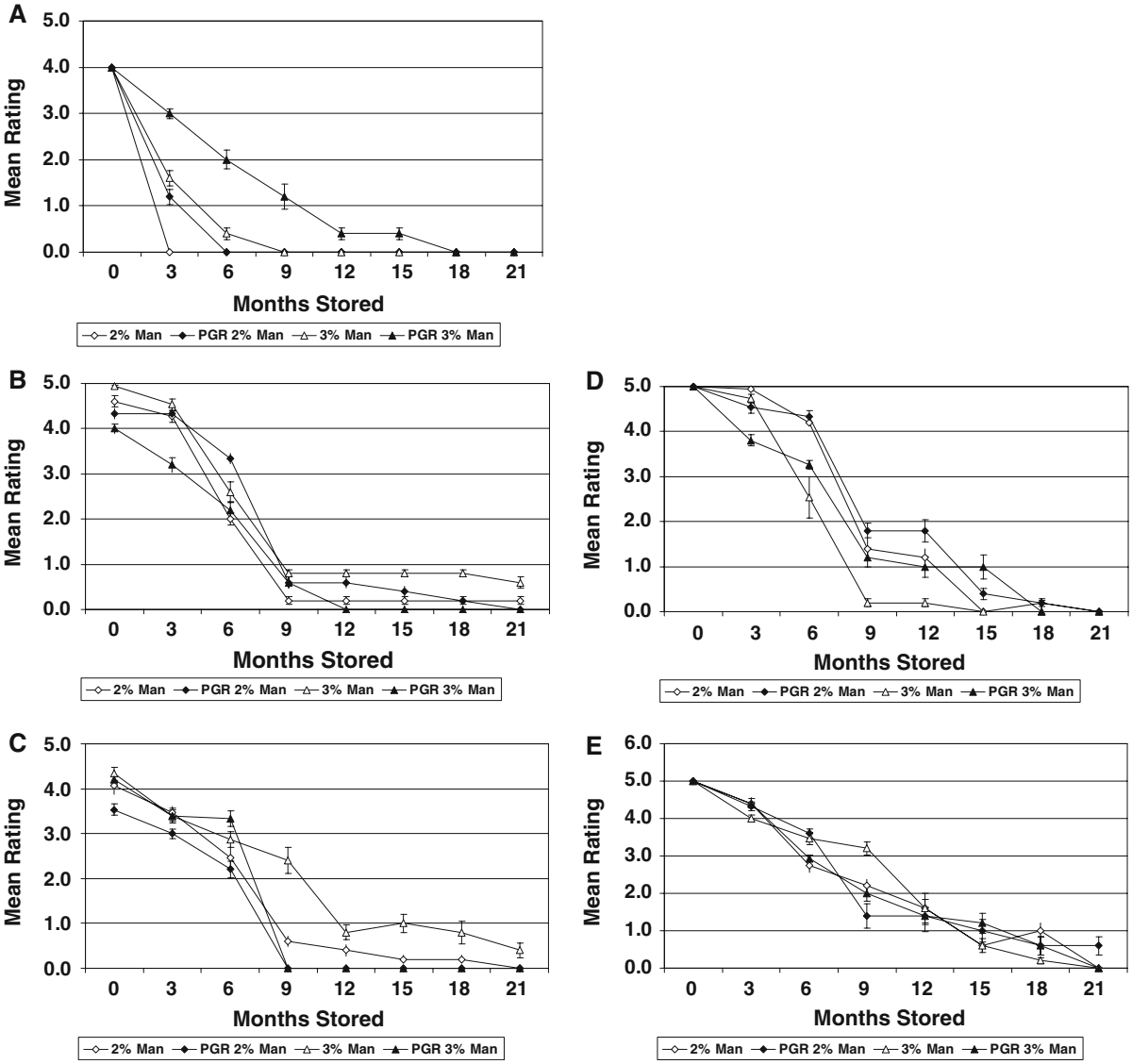

Fig. 3 Mean condition ratings (±standard error) of shoots stored on MS medium with 2 or 3% mannitol, with or without PGRs for 21 months. 'Grushovka Vernenskaya' shoots in (**a**) tissue culture bags (**b**) tubes, and (**c**) jars or TM-6 shoots in (**d**) tubes or (**e**) jars. $n = 15$

were ultimately used as the other techniques showed no variation. Analysis of cold stored plants was done with 4 primers that produced moderate banding patterns (Table 1).

Polymorphism of nucleotide sequences between separate samples of DNA was defined by presence or absence of the concrete bands in spectrum of DNA fragments at electrophoresis. The absence of the band in a sample is probably explained by changes in one or both sites of primer binding or is conditioned by deletion of the DNA fragment, carrying these sites. Deletion or insertion inside of amplified fragment of DNA-matrixes leads to changes of the amplification product size. We optimized and strictly controlled the condition of the reactions to ensure repeatability. We saw no major differences using this technique (Fig. 6). Some minor differences were seen with the first primer (Fig. 7a, b) but the other three primers showed equivalent banding (Fig. 7c–e). These preliminary investigations indicate that there is genetic stability in these samples after long-term cold storage.

Discussion

In vitro cold storage of temperate fruit crops is a useful technique for germplasm preservation and exchange. Storage facilities, growth media and storage containers vary from institution to institution making comparisons of techniques difficult. Optimizing the conditions of storage for a particular facility can greatly improve the storage

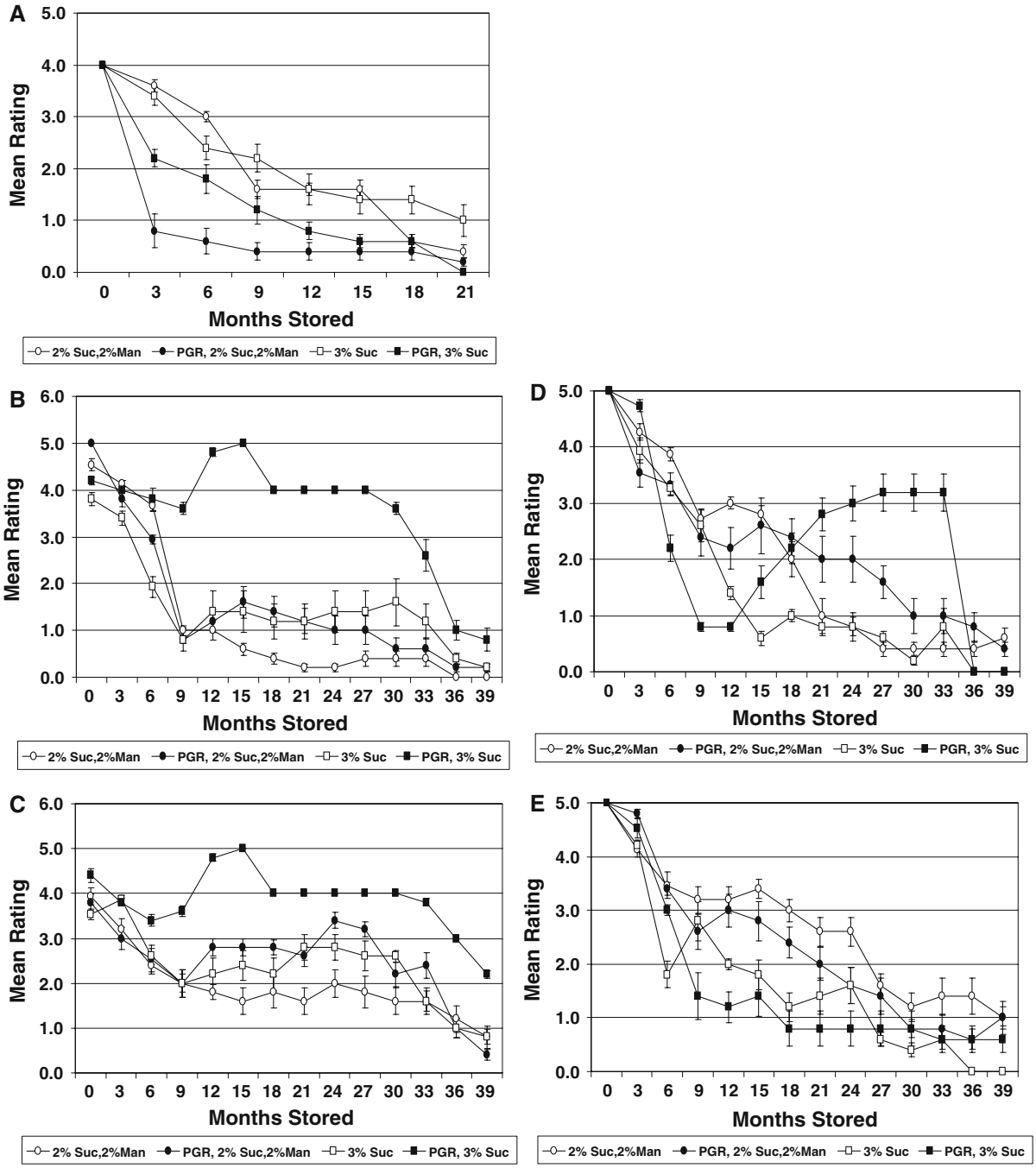

Fig. 4 Mean condition ratings (±standard error) of shoot cultures stored on MS medium with 3% sucrose or 2% sucrose and 2% mannitol combined, with or without PGRs for 39 months. 'Grushovka Vernenskaya' shoots in (**a**) tissue culture bags (**b**) tubes, and (**c**) jars or TM-6 shoots in (**d**) tubes or (**e**) jars. $n = 15$

potential and increase the security of important plant collections. Apple cultures were first stored by Lundergan and Janick (1979) in tubes for 1 year at 1 or 4°C. In other cases 4°C was used for storage of apple shoots in tubes and storage lasted up to 48 months (Orlikowska 1992a). The only report of apple storage in tissue culture bags indicated a 9 month storage period for 'M26' clone NAKB virus-free shoot cultures (Lambardi et al. 2006).

The initial part of our study focused on screening a range of apple germplasm for storage in tissue culture bags on standard medium with no PGRs. There were three general responses to the cold storage. Storage was good for up to 14 months for 10 of the 16 genotypes, but for less than 1 year for the others (Fig. 1). We considered that the native cold hardiness of the genotypes might translate into similar results in cold storage, but this did not seem to be

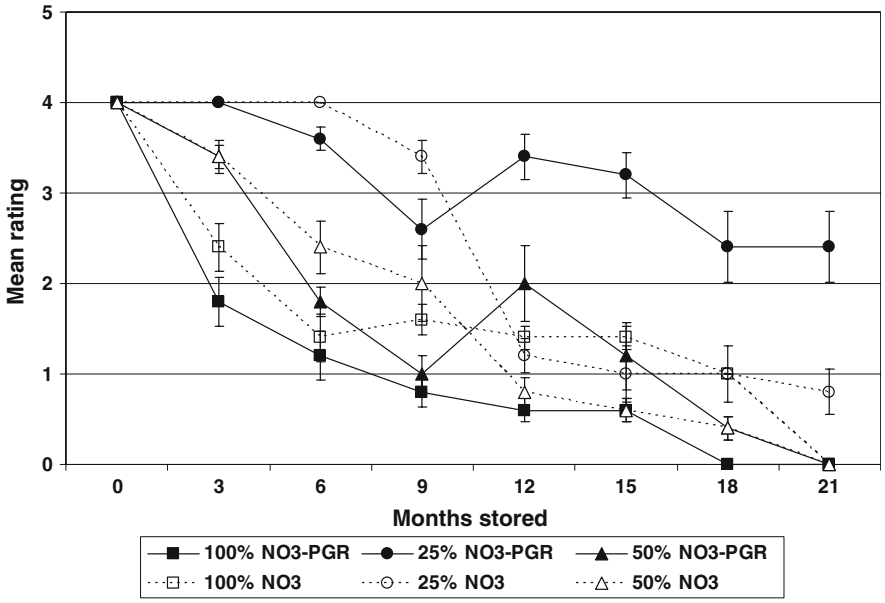

Fig. 5 Storage of 'Grushovka Vernenskaya' for up to 21 months in tissue culture bags on MS medium with 3% sucrose and 25, 50 or 100% of the normal NO_3 concentration. Cultures were tested with PGRs (closed symbols) (0.5 mg mgl^{-1} BA and 0.1 mg mgl^{-1} IBA) and without PGRs (open symbols). Mean condition ratings (±standard error). $n = 15$

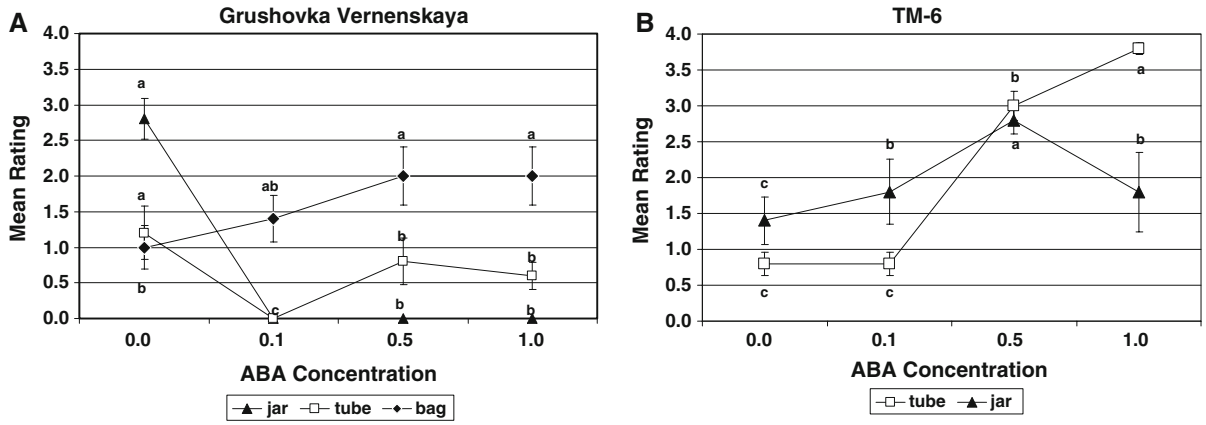

Fig. 6 The effect of container and ABA concentration (mgl^{-1}) on storage ratings of apple shoot cultures stored on MS medium with 3% sucrose for 21 months (**a**) 'Grushovka Vernenskaya' and (**b**) wild apple selection TM-6. Mean condition ratings (±standard error). $n = 15$. Points on the same line followed by a different letter are significantly different ($P < 0.001$)

Table 1 Sequence of primers used for comparing in vitro cultures with 39 month cold-stored samples tested for apple polymorphisms

Number by order	Sequence
2.3	CCTGAGGTAC
2.6	GCGTAGCGTT
2.12	GCGCTTATCG
2.14	GTCCGATAGC

the case. The genotypes 'Agat', 'Nurgul', 'Voskhod', and TM-6 are very cold hardy under field conditions (Nurtazina N.Y., Kazakh Institute of Horticulture and Viticulture, personal communication) while 'Royal Red Delicious' has medium hardiness and 'Grushovka Vernenskaya' has low field cold hardiness. Kushnarenko et al. (2008) studied the cold hardiness of in vitro shoots and found that with 1 week of cold acclimation the LT_{50} (temperature at which 50% of the shoots are killed) of 'Voskhod' shoots reached −15°C and TM-6 shoots reached −12°C with maximum hardiness of −14°C at 3 weeks. 'Royal Red Delicious', 'Golden Delicious' and 'Grushovka Vernenskaya' ranged in LT_{50} from −11 to −10°C at 1 week and reached their maximum cold hardiness of −12°C after 3 weeks CA. However, the in-vitro cold hardiness of the genotypes did not appear to correlate with storage ability or with the three storage responses. It appears that a 3-week cold acclimation period would be optimum for apple cultivars. It may also be that a colder storage temperature such as 1°C would be more effective for in vitro apple storage as was seen for *Ribes* germplasm (Gunning and Lagerstedt 1985). It will be useful to screen these genotypes on the best treatments in this study with a 3-week cold acclimation to see if storage

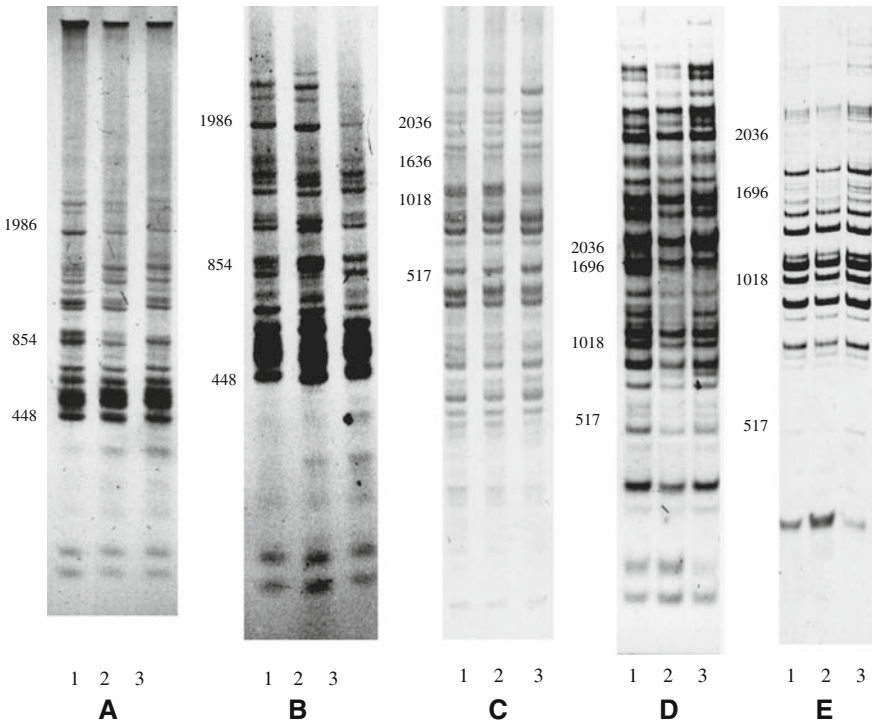

Fig. 7 Electropherograms of amplification products with one primer for 'Grushovka Vernenskaya' (**a**) and four primers for TM-6 (**b**, **c**, **d**, **e**): 1–initial sample, 2, 3 after 39 months of cold storage

can be extended to 2 or more years as is possible for pear (Reed and Chang 1997).

The carbon source used for storage was significant for both of the genotypes tested. MS medium with 3% sucrose and no PGRs was considered the control treatment based on successful storage of other temperate fruit crops (Reed 1993, 1999; Reed et al. 1998). In those studies, blackberries and pears could be cold stored in tissue-culture bags for 9–39 months depending on the genotype. When 'Grushovka Vernenskaya' was stored in bags on combinations of carbon source and PGRs, the MS medium with 3% sucrose and no PGRs resulted in longer storage of the shoots (>9 months) compared to the other treatments (Figs. 3, 4). Medium with 2% sucrose and 2% mannitol was the next most successful medium with storage lasting for about 9 months. Mannitol alone was not suitable for storage of either genotype (Fig. 3). This is similar to the results found a recent study where 'M26' apple rootstocks could be stored on medium with 4% mannitol for only 3 months in jars and less in tissue culture bags (Lambardi et al. 2006).

Storage of 'Grushovka Vernenskaya' in tissue culture bags was significantly affected by the amount of nitrates in the medium (Fig. 5). Plantlets on 25% of the MS concentration of NO_3 and 3% sucrose with PGRs stored significantly longer than those on higher nitrate concentrations (21 months compared to <12 months). This increase in storage time with a reduction in the nitrogen concentration is similar to that shown in *Vitis* and *Rubus* shoots stored at growth room temperatures (Moriguchi and Yamaki 1989; Reed 1993).

Storage in tubes and jars on the same combinations of carbon source and PGRs showed similar but not identical results for 'Grushovka Vernenskaya' and TM-6. Overall, tubes with 3% sucrose and PGRs were the best alternatives for both genotypes and allowed 33 months of cold storage without transfer. This compares favorably to the best storage of 'Moscatella' (14 months) and 'Starkspur Red' (18 months) on half-strength MS with 3% sucrose and PGRs (Negri et al. 2000). Storage of plantlets with the addition of ABA to the 3% sucrose medium showed varied results (Fig. 6). The two genotypes differed in response to ABA when stored in jars and tubes. 'Grushovka Vernenskaya' shoots were best without ABA while TM-6 shoots were in significantly better condition with 0.5 mg mgl^{-1} ABA than the other treatments. Negri et al. (2000) found that low BAP and ABA produced the poorest storage for two apple genotypes while moderate BAP with or without ABA was successful for 14–18 months.

The limited RAPD analysis of 'Grushovka Vernenskaya' and TM-6 before and after cold storage showed no changes in the DNA after storage for 39 months (Fig. 7). Hao and Deng (2003) studied the genetic stability of 'Gala' apple stored for 1 year and found some changes in methylation, but no differences in AFLP patterns. Although somaclonal variation is greatest in plants regenerated from callus or suspension cultures, it can also occur in micropropagated plants, especially if shoots develop from basal callus. Techniques to determine the genetic stability of plants may include evaluation of the plant phenotype through field evaluation of the plants, cytological,

biochemical, or molecular evaluations. Evaluation of plants after cold storage is rare, but there are many cases of phenotypic evaluation of tissue culture plants and molecular evaluation of cryopreserved plants. Harding (2004) reviewed available forms of analysis for cryopreserved plants. The literature on phenotypic variation indicated that some in vitro plantlets show temporary abnormalities that do not persist when the plants are grown in soil. Harding (2004) noted that in most cases cited in the literature, cryopreserved plants showed few if any morphological changes; but most of these studies did not include a detailed examination of descriptors. Molecular studies, either with RAPDS, RFLP analyses and DNA-DNA hybridization as used in this study, can have their own problems; mostly that each of these techniques examines only a small part of the genome and thus is not likely to detect genetic instability. It is likely that both genetic analysis and morphological analysis are needed to determine genetic stability of stored plants.

Overall it appears that cold storage of apple shoot cultures of multiple genotypes is successful for more than 21 months in tissue culture bags with either 25% MS nitrate nitrogen, or 0.5 mg mgl^{-1} ABA without PGRs, or for 33 months in jars or tubes on standard MS with 3% sucrose and PGRs. It is possible that a longer cold-acclimation period would also extend storage of apple germplasm.

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